AD_____

GRANT NUMBER DAMD17-96-1-6085

TITLE: Identification of Components of the Cell Death Pathway

PRINCIPAL INVESTIGATION: Vishva Dixit, M.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48109

REPORT DATE: July 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1997	Annual (1 Jun 96 - 31 May 97)

4. TITLE AND SUBTITLE
Identification of Components of the Cell Death Pathway

5. FUNDING NUMBERS
DAMD17-96-1-6085

6. AUTHOR(S)
Vishva Dixit, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Michigan
Ann Arbor, Michigan 48109

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Programmed cell death has been shown to play a role in breast involution, morphogenesis and cancer. The effector arm of the cell death pathway is a family of cysteine proteases related to interleukin-1 converting enzyme that has recently been renamed caspases. We have determined for the first time the sequential steps involved in the death pathway engaged by a cell surface receptor (CD-95/Fas/Apo-1). A pivotal role in this process is played by FLICE, a new receptor associated Caspase. This protease directly associates with the receptor and its substrate specificity is compatible with the hypothesis that it is the protease at the apex of a Caspase cascade. We show that the activation of FLICE can occur autocatalytically due to the remaining proteolytic activity of the zymogen form. FLICE2, a second member of the receptor associated Caspases was also cloned and characterized. Further, viral inhibitors of apoptosis were identified that specifically target the FLICE activation step. While the FLICE Caspases are likely to be also involved in TNFR-1 signalling a second death pathway emanating from this receptor was identified that acts through the new adapter molecule RAIDD which recruits Caspase-2 to the receptor.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	23
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

VMD Where copyrighted material is quoted, permission has been obtained to use such material.

VMD Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

VMD Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

VMD In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

VMD In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Vilma Pixt 6/26/97

PI - Signature Date

Table of contents

Front Cover	1
Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Body	6
Molecular cloning and characterization of FLICE/ Caspase 8	6
Molecular cloning and characterization of FLICE2/ Caspase 10/b	12
Characterization of a novel family of viral inhibitors of apoptosis	14
Molecular cloning and characterization of RAIDD a new 'death' adapter molecule	16
Conclusion	19
Publications supported by the grant	20
Meeting abstracts	21
References	22

Introduction:

Programmed cell death (PCD) or apoptosis is now recognized as a fundamental cellular process. The elucidation of the molecular mechanisms involved in this process has great potential to lead to new drug targets and treatments for a variety of cellular disorders including breast cancer. Current breast cancer treatments such as ionizing radiation and chemotherapy already rely on the induction of apoptosis. Understanding the molecular events induced by these therapies will lead to avenues for improving the efficacy and specificity of these treatments.

The objective of the grant application was to delineate in biochemical terms components of the death pathway, especially cysteine proteases belonging to the Caspase family that form the effector arm of the death pathway. We have focused our studies on the activation of these Caspases by signals emanating from the death receptors Fas (CD-95/Apo1) and TNFR-1.

The discovery that the interleukin-1 converting enzyme is a homologue of the *C. elegans* ced3 gene product led to molecular cloning and characterization of a whole family of proteases now designated as Caspases. Our lab was involved in the discovery of several of these Caspases.

A year ago the burning question was: How are Caspases activated? It became evident early on that the Caspase regulation did not occur at the transcriptional level. Caspases are expressed as zymogens and are activated by proteolytic processing. The question therefore reduced itself to: How is the proteolytic processing of Caspases initiated?

This progress report will show the substantial progress made towards answering this question in particular for FAS signaling. We have shown that one class of Caspases (FLICE) directly associate with the receptor. Further, the investigation of the substrate specificity of the receptor associated Caspases suggests the existence of a Caspase cascade. Insights into the molecular activation mechanism were obtained by using an in vivo crosslinking approach. In addition, viral inhibitors of this process were also identified in our lab during the last year.

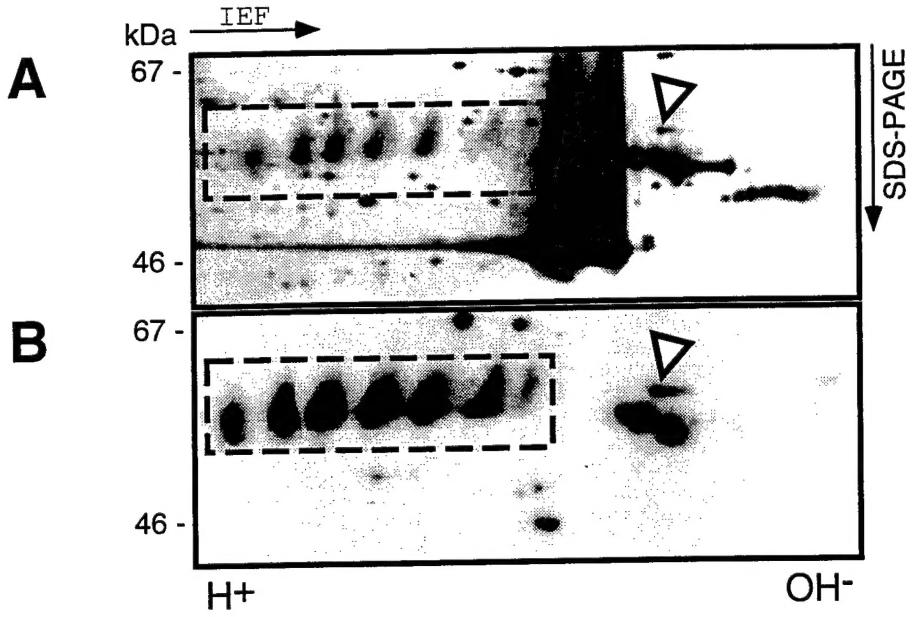
Death signaling from the TNFR-1has similar components as the FAS signaling pathway. Our discovery of the adapter molecule RAIDD shows that additional, perhaps redundant, pathways mediate the TNF death signal.

Body:

1) Molecular cloning and characterization of FLICE/ Caspase 8

At least four endogenous proteins, CAP1-4, associate with activated CD95 to form the death induced signaling complex (DISC). CAP1 and CAP2 have been identified as different forms of the previously isolated FADD/MORT1 (8). Subsequent dominant-negative studies have established that endogenous FADD is essential for the recruitment of CAP3 and CAP4 to the CD95 DISC (3). To identify CAP4, we utilized nano-ES MS/MS to generate peptide sequence from gel-isolated protein (16)(Figure 1).

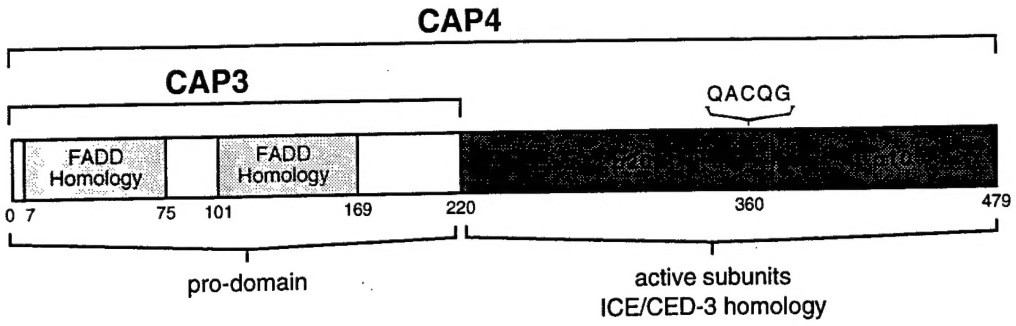
Figure 1



A) Silver-stained 2D IEF/10% SDS-PAGE of immunoprecipitated (anti CD95) death inducing signaling complex (DISC) from K50 cells.
B) Autoradiography of an analysis of metabolically labeled DISC proteins on a 2D IEF/10% SDS-PAGE gel. Migration position of CD95 spots are labeled by stippled box. The migration position of CAP4 is indicated by an arrowhead.

The CAP4 spot contained approximately 0.5 pmol of protein. The complete sequence of five peptides, covering a total of 41 amino acid residues and two partially sequenced peptides that could be used as sequence tags, was determined. Homology searches against the Human Genome Sciences cDNA database revealed a 3.0 kb cDNA with a 1437 bp open reading frame that encoded a novel protein with a predicted molecular mass of 55.3 kD and a pI of 4.91, consistent with the size and pI of CAP4, as determined by 2D gel analysis (Figure 1A and 1B). Sequence comparisons of the cDNA, designated FLICE, had substantial homology to both FADD and Caspase family of cysteine proteases. CAP3 was also subjected to the same analysis and was found to be a protein identical to the N-terminus of FLICE (Figure 2).

Fig. 2

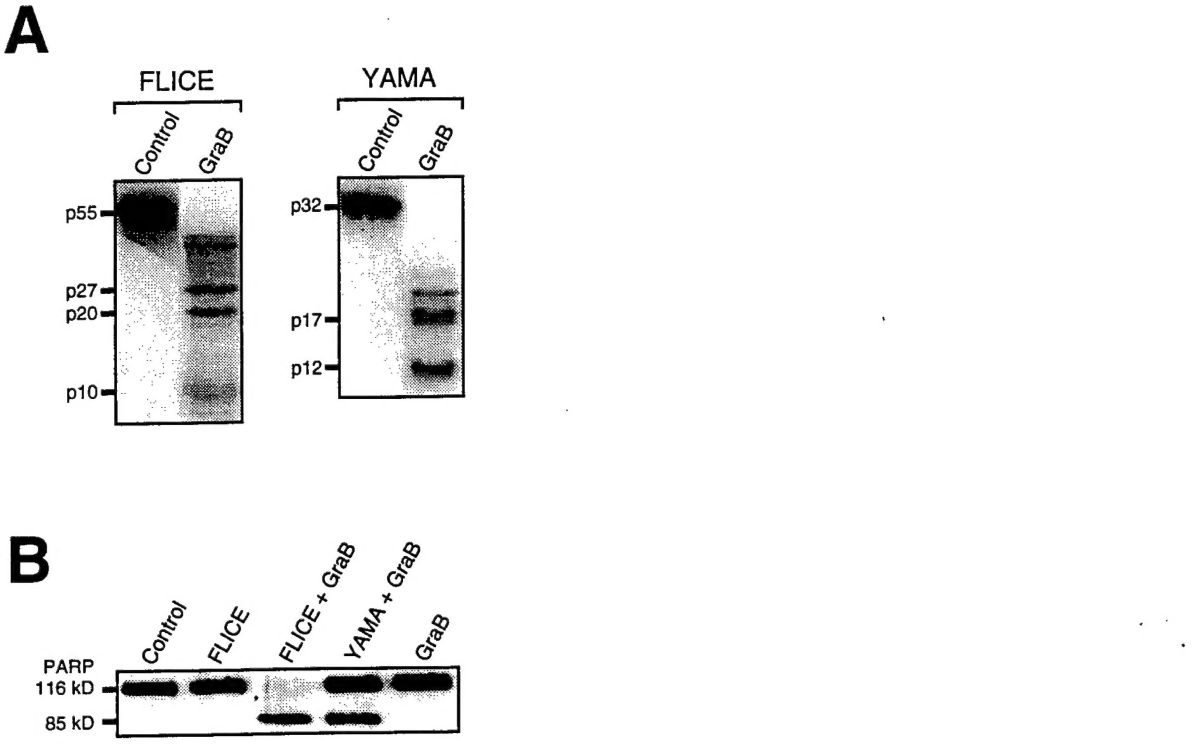


Schematic model of the FLICE protein.
The N-terminal portion of FLICE (prodomain) contains two domains that show marked homology to the N-terminal DED domain of FADD (shaded boxes). The C-terminus of FLICE encodes a novel member of the ICE/CED-3 family of cysteine proteases (filled boxes). The pentapeptide QACQG is indicated.

Previous studies show that FADD-DN, missing the death effector domain (DED) blocks the recruitment of CAP4 to the DISC, suggesting that the DED is an essential component (3). To determine whether FLICE can directly bind FADD and whether the DED of FADD is necessary for this binding, in vitro transcribed and translated FLICE was precipitated with His-6-tagged FADD and His-6-tagged FADD-DN immobilized onto Ni2+ beads. As predicted, FLICE bound full-length FADD but not FADD-DN . Further confirmation of these results were obtained *in vivo* by cotransfection of 293T cells with FLICE and epitotpe tagged FADD. These experiments establish that the DED is a homophilic protein interaction domain.

Members of the ICE/CED-3 gene family are synthesized as proenzymes and can be activated by in vitro by Granzyme B. Thus we determined whether FLICE could serve as a substrate for Granzyme B and, more importantly, whether FLICE could function as a cysteine protease. In vitro transcribed and translated FLICE and Yama (Caspase 3) were incubated with purified Granzyme B. After 4 hr at 37°C, FLICE and Yama were proteolytically processed, generating putative active p20/p10 and p17/p12 subunits, respectively (Figure 3A). Next, we assessed whether Granzyme B-mediated (GraB) cleavage of FLICE generated an active enzyme by assaying for poly (Adp- ribose) polymerase (PARP) cleavage. PARP is proteolyzed during many forms of apoptosis, and the enzyme or enzymes responsible likely belong to the ICE/CED-3 family (11). To exclude the possibility of direct cleavage of PARP by GraB, GraB-processed FLICE and the positive control Yama (Caspase 3) were incubated with a selective inhibitor of GraB (anti-GraB). Both GraB-processed Yama and FLICE were active, as determined by their ability to cleave PARP (Figure 3 B). Therefore, unlike ICE, FLICE and other members of the CED-3 subfamily are able to cleave PARP into signature apoptotic fragments (14).

Fig. 3

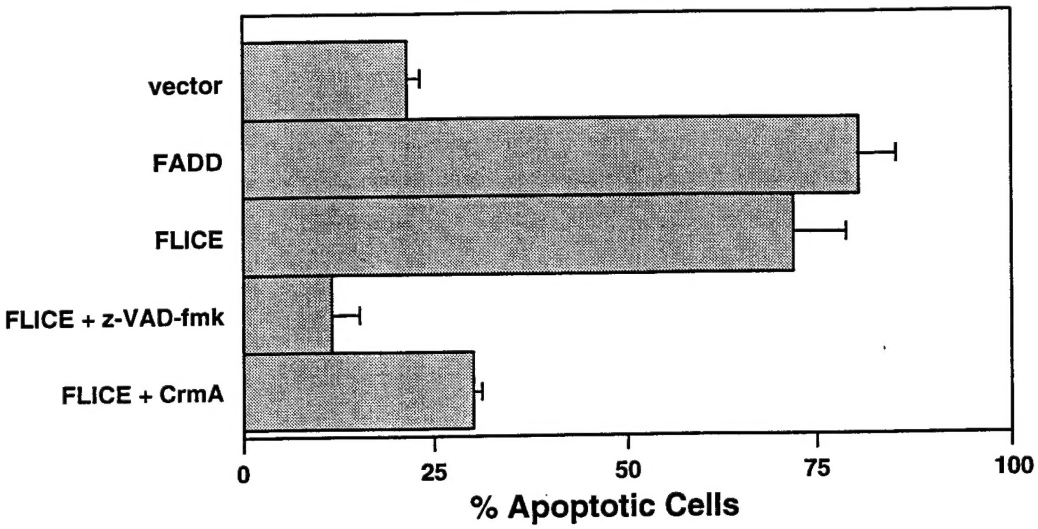


Granzyme B-processed FLICE is an active protease that cleaves PARP to the signature 85 kDa apoptotic fragment.

A) Processing of FLICE and YAMA by Granzyme B (GraB) in vitro. ^{35}S-labeled FLICE and Yama were incubated with purified GraB for 4 hr and the reaction products analyzed by SDS-PAGE and autoradiography. The active subunits of YAMA (p17/p12) and FLICE (p20/p10) are indicated. A27 kDa species of FLICE was also generated by granzyme B, likely representing the FLICE prodomain. B) GraB-processed FLICE and Yama cleave PARP.. Following GraB processing, anti-Grab, a specific GraB inhibitor, was added and proteolytically processed FLICE or Yama assessed for activation detectable when PARP was incubated alone (control) or with the zymogen-unprocessed form of FLICE. PARP was detected by immunoblotting using a specific anti-PARP antibody.

To study the functional role of FLICE, we transiently transfected MCF-7 breast carcinoma cells with an expression vector encoding the FLICE protein and subsequently assessed for apoptotic features. Like the other ICE/CED-3 family members, overexpression of FLICE caused apoptosis (Figure 4). The FLICE-transfected cells displayed morphological alterations typical of adherent cells undergoing apoptosis, becoming rounded, condensed, and detaching from the dish (data not shown). FLICE-induced cell death was inhibited by the broad spectrum ICE inhibitor z-VAD-fmk (5, 10), which is also a potent inhibitor of CD95-induced apoptosis. Like the peptide ICE family inhibitor, CrmA blocked FLICE-induced cell death (Figure 4), suggesting that FLICE may be a physiologic target for this pox virus serpin.

Fig. 4

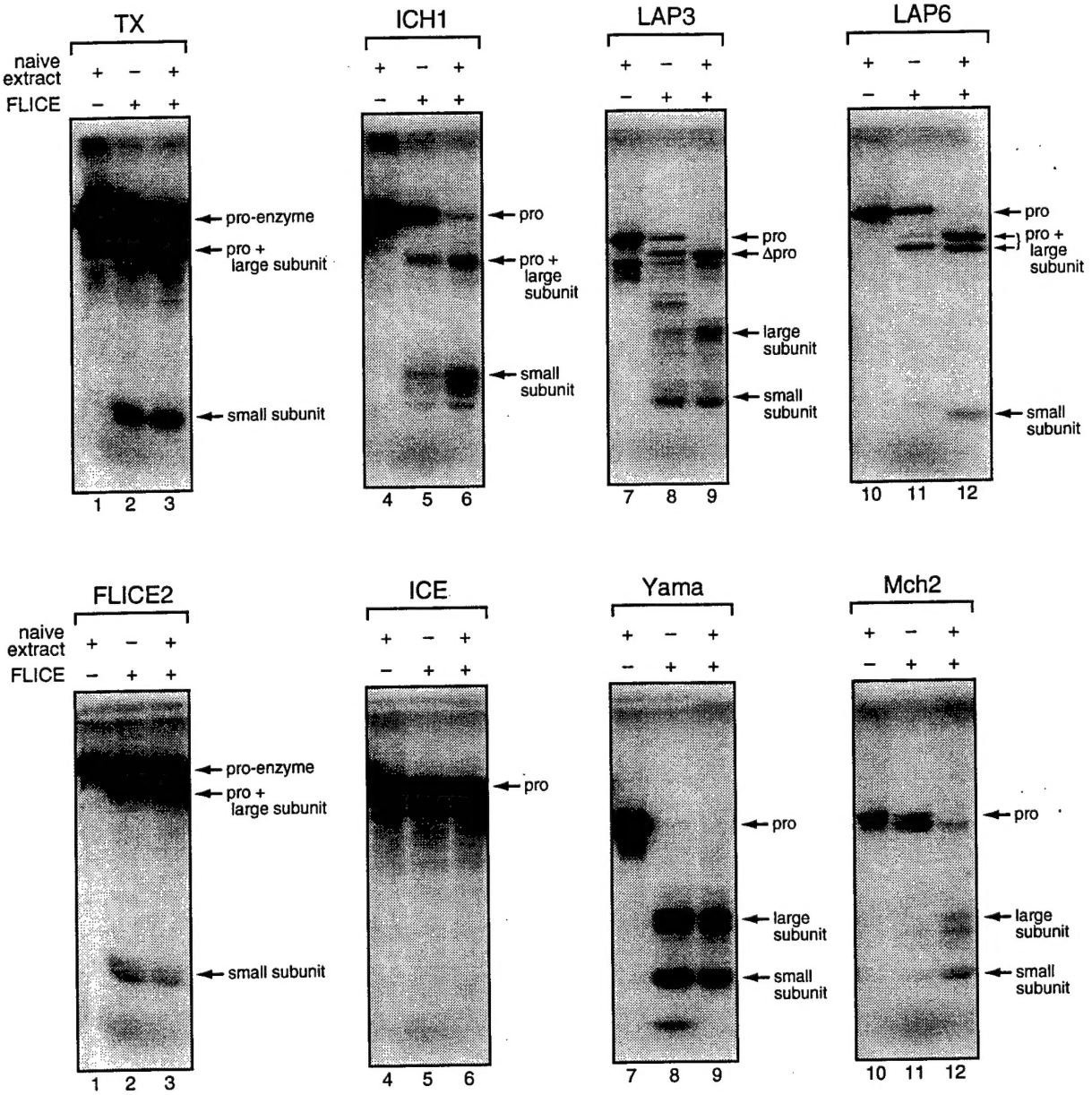


FLICE-induced apoptosis is blocked by ICE/CED-3 inhibitors z-VAD-fmk and CrmA. *Previously characterized MCF-7 clonal lines were transiently transfected with pCMV-β-galactosidase in the presence of a two fold excess of vector alone or expression constructs encoding FADD or FLICE. The broad spectrum ICE family inhibitor z-VAD-fmk (20 μM) was added to the cells 5 hr posttransfection. Cells were fixed and stained for β-Gal expression 42 hr following transfection. The data (mean plus or minus standard error of the mean) shown are the percentages of round blue cells as a funciton of the total number of blue cells counted (n=3).*

To study the enzymatic properties of this new Caspase we expressed recombinant Flice in bacteria as a 6 His tagged protein. We showed that purified protein is able to induce apoptosis in a cell-free system (9). The ability of FLICE to cleave other members of the Caspase family was assayed by incubation of the recombinant protease with in vitro transcription translated Caspases. These assays were done in the absence or presence of cytosolic extract to identify direct or indirect processing respectively (Fig. 5). As expected from a Caspase that is at the top of the cascade it was able to cleave directly Yama (Caspase 3), LAP3 (Caspase 7), LAP6 (Caspase 9) and FLICE2 (Caspase 10/b). In contrast, MCH2 (Caspase 6) and ICH1 (Caspase 2) were efficiently cleaved only in the presence of naive extract. These results are consistent with the requirement of an intermediary cytosolic component for FLICE-mediated activation of Mch2 and ICH1.

Recombinant Flice was also used to investigate the inhibitor profile of this Caspase. It was highly sensitive to the tetrapeptide inhibitor aldehyde DEVD but not YVAD. It was also inhibited very efficiently by recombinant CrmA, suggesting that FLICE may be the physiological target of CrmA.

Fig. 5

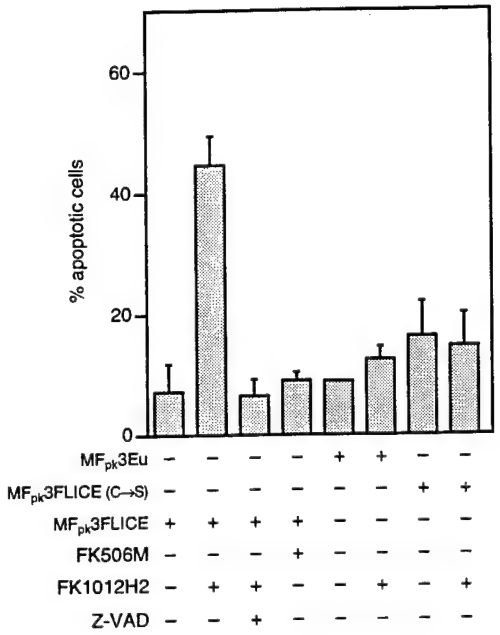


Differential activation of ICE-CED3 family members following addition of FLICE to naive extracts.

B) Radiotracer analysis of the activation of Tx, ICH1, LAP3, LAP6, FLICE2, ICE, YAMA, and Mch2 pro-enzymes (lanes 1,4,7,and 10) in the presence (lanes 3, 6, 9, and 12) or absence (lanes 2, 5, 8, and 11) of naive extract incubated with recombinant FLICE for 2 h at 30 ^{0}C.

A in vivo crosslinking method was used to address the question on how FLICE is activated after recruitment to the receptor(1, 4, 6). Chimeric expression constructs were made in which the prodomain of FLICE was replaced by a myristilation signal followed by three FK1012 H2 binding sites (Fpk3). FK1012 H2 is divalent ligand that can crosslinks two Fpk containing molecules. We were able to mimic recruitment to the membrane and oligomerization of FLICE upon addition of FK1012 H2. As shown in Fig. 6, 293 cells, upon addition of the corsslinker, underwent apoptosis.

Fig. 6



FK1012H2 induces oligomerization of FLICE triggers self-processing and apoptosis.
(D) 293 cells were transiently transfected with $MF_{pk}3E_U$, $MF_{pk}3E_U3FLICE$, or $MF_{pk}3E_U3FLICE$ (C->S) and pCMV-β-Gal. 36 hours post transfection cells were treated with FK1012H2 (250 nM) or FK506M (250 nm) with or without the addition of the broad-spectrum caspase inhibitor z-VAD-fmk (20μM). After 3 hours cells were fixed, stained with X-Gal and examined by phase contrast microscopy. Data (mean+/- s.e.m.) shown are the percentage of round and blebbing apoptotic cells as a function of total number of blue cells counted (n>3).

FK1012 H2 induced cell death was mediated by the proteolytic activity of FLICE, as demonstrated with the active site cysteine mutant chimeric construct which has no apoptotic activity in the presence of FK1012 H2. In addition, the killing is inhibited by z-VAD, a broad spectrum Caspase inhibitor.

These results imply that the high local concentration of FLICE generated upon recruitment to the receptor causes self activation. In support of this hypothesis is also the observation that a FLICE molecule with a mutation at the zymogen cleavage site mutated still retains proteolytic activity.

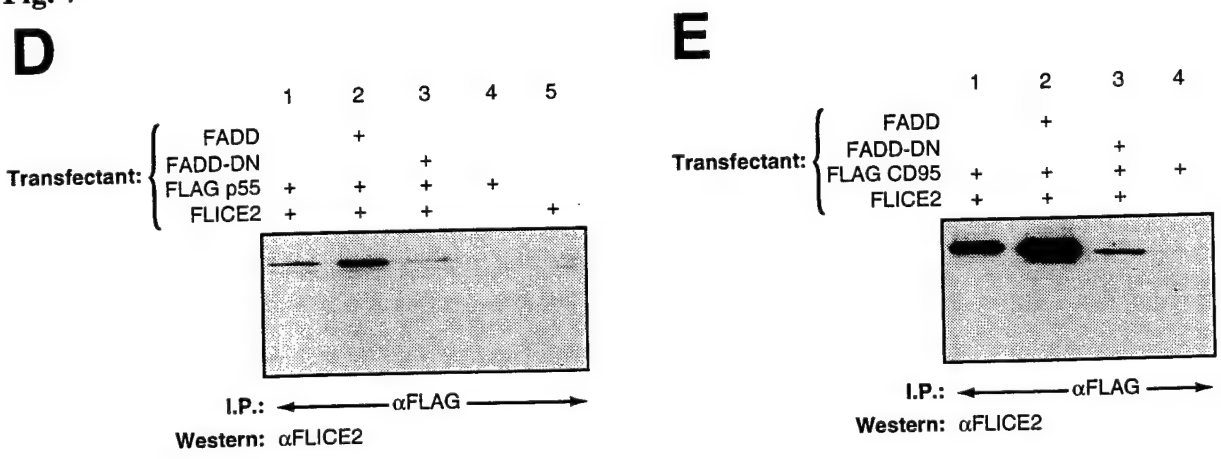
We are currently in the process of generating monoclonal antibodies towards FLICE. The goal is to produce reagents that can detect the endogenous molecule. With such a reagent in hand we can address the question of whether FLICE is activated only during FAS signaling or whether other apoptotic stimuli also involve FLICE activation.

2) Molecular cloning and characterization of FLICE2/ Caspase 10/b

A search of the GenBank EST database revealed a clone T96912 derived from a human fetal spleen library with high homology to the conserved GSW sequence contained within the small catalytic subunit of all Caspases. Sequencing of the EST clone revealed a downstream stop codon as well as 1.3 kb of 3' untranslated region followed by a poly (A) tail. Full length sequence was obtained by two rounds of PCR extension employing vector and gene specific primers. The derived open reading frame encoded a protein of 521 amino acids with a molecular weight of 59 kD. Because of its significant homology over its entire sequence (28% identity) to FLICE/MACH it was designated FLICE2.

FLICE2 was shown to be a protease belonging to the Caspase 3 family of proteases by using in vitro assays as described above for FLICE. Because FLICE2 also contains two death effector domains at the N-terminus, we investigated if this domain was also able to participate in homophilic interactions. We show that FLICE2 binds specifically to the death domain of FADD *in vitro* and *in vivo*. Further, we were able to show that FLICE2 is recruited to FAS and TNF receptors through endogenous FADD (Figure 7)

Fig. 7

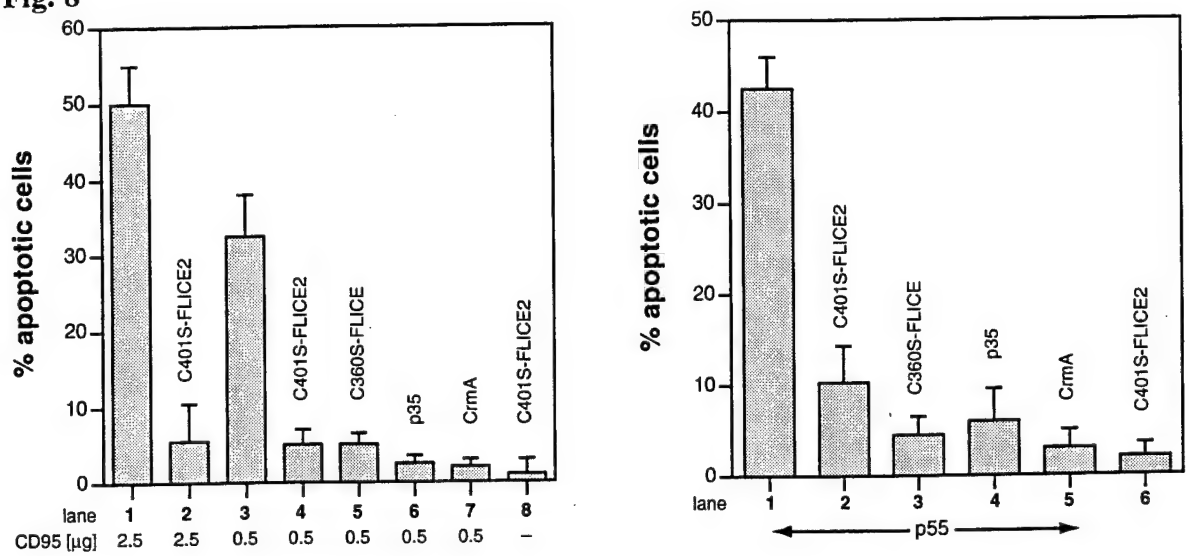


FLICE2 binds through FADD to the death receptors CD95 and p55.
D&E, 293 cells were co-transfected with the indicated expression constructs. Cell lysates were prepared after 40 h, followed by immunoprecipitation with FLAG antibodies and immunoblotting with an antibody specific for the small catalytic subunit of FLICE2.

As shown, FLICE2 bound both death receptors and a substantial increase in binding was observed when FADD was included in the transfections (Fig. 3 D&E, Lanes 1,2). This was consistent with initial binding being mediated by endogenous FADD and being enhanced by the expression of exogenous FADD. Confirming this was the finding that expression of FADD-DN which lacks a DED and is therefore unable to bind FLICE or FLICE2 attenuated the association of FLICE2 with the death receptors (Fig. 7 D&E, Lane 3).

FLICE2 not only associates with the death receptors but an active site mutant of the protein behaves as a dominant negative for TNF R1 and FAS signaling (Figure 8).

Fig. 8



Dominant negative FLICE2 protects from death receptor and FLICE2 overexpression induced apoptosis.
293 and 293EBNA cells were co-transfected with the indicated constructs and pCMV-βGAL. Cells were fixed and stained with X-Gal. The fraction of apoptotic blue cells was evaluated by microscopy.

293EBNA cells underwent apoptosis when transiently transfected with CD95 receptor (Fig. 8). This auto-activation on overexpression occurred in a dose dependent manner (lanes 1, 3). Co-transfection with the active site FLICE2 cysteine mutant effectively inhibited the induction of apoptosis to the same extent as CrmA or p35 (lanes 6,7). Similarly, transfected cells overexpressing the p55 receptor underwent an apoptotic demise by 24 h. Again, expression of the FLICE2 active site mutant inhibited apoptosis to the same extent as p35, CrmA, and the active site mutant of FLICE (C360SFLICE). Taken together, these results are in keeping with the involvement of FLICE2 in the death pathway engaged by both CD95 and p55. Additionally, these results are consistent with FLICE2 operating at the apex of the Caspase cascade.

Even though FLICE and FLICE2 show a high degree of sequence conservation we predict that the substrate specificity of the two molecules to be different. This is based on the analysis of the amino acids that are predicted to contact the P4 residue of the Caspase substrate. These predictions were made using the data from the crystal structure of Caspase 3 and 1(12, 15).

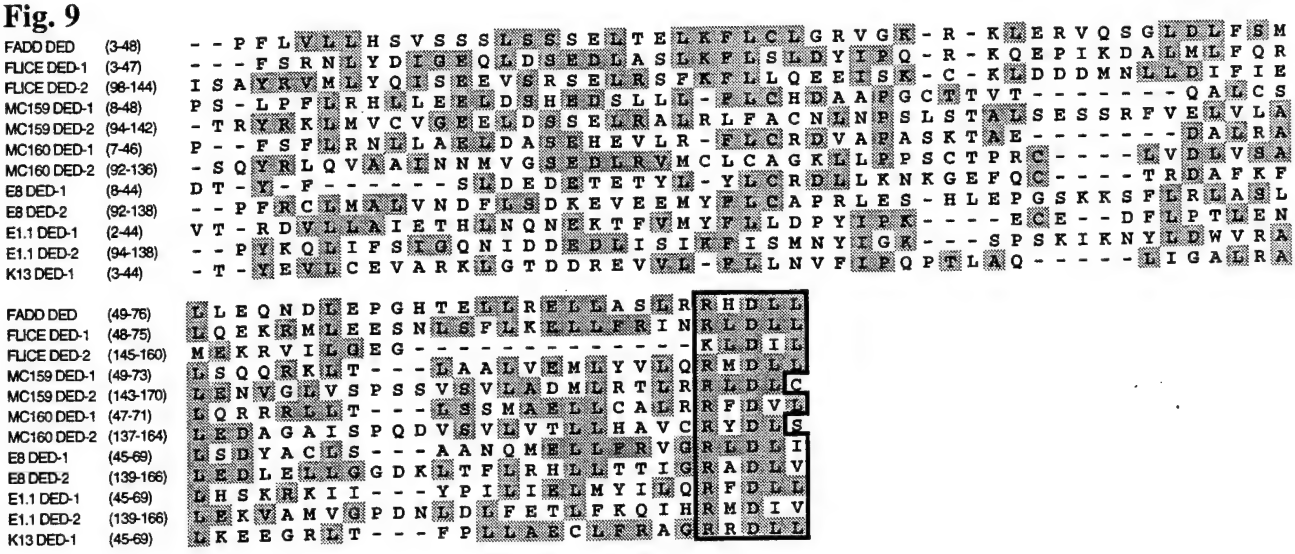
In order to undertake these experiments we need to recombinant active FLICE2. First attempts using the same strategy for expression as was used for FLICE did not yield an active enzyme. We are currently investigating if coexpression of FLICE2 with the bacterial chapersonin proteins GroEL and GroES will result in an active recombinant protein.

Further, as with FLICE, we are generating monoclonal anti FLICE2 antibodies to be able to assess the association and activation status of endogenous FLICE2. In particular, this reagent would allow to test the hypothesis that receptor oligomerization activates the Caspase cascade by approximating the two FLICEs such that they act as substrates for each other.

3) **Characterization of a novel family of viral inhibitors of apoptosis**

The availability of several DED containing sequences facilitated homology searches for other proteins with this sequence motif. We identified several viral proteins that contained this sequence motif (Fig.8).

Fig. 9

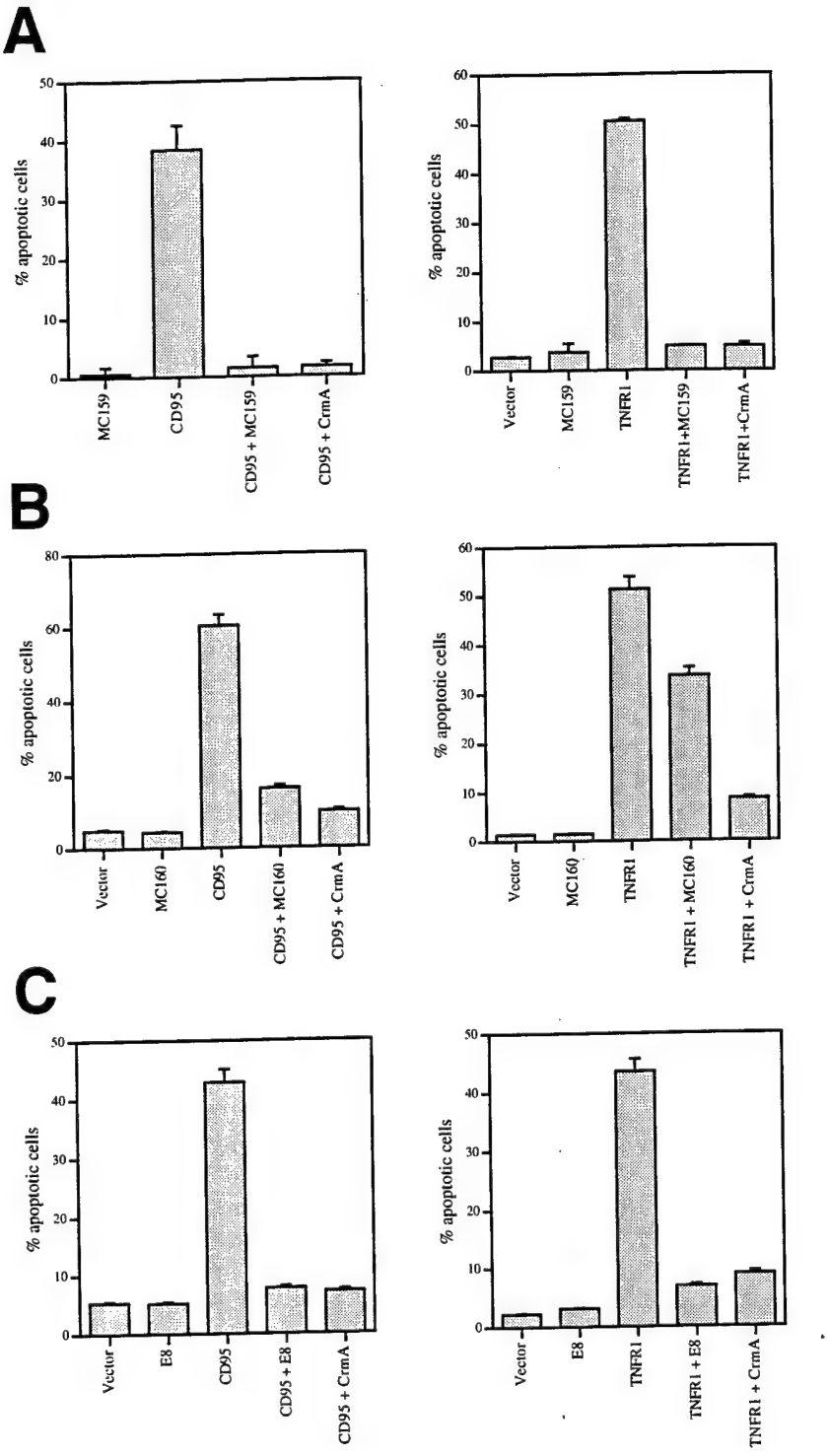


Sequence alignments of viral DED-containing molecules.
The DED motifs contained within the adapter molecule FADD and the initiating caspase FLICE were aligned to DED-like motifs present in the molluscum contagiosum virus proteins MC159 and MC160, bovine herpesvirus 4 protein E1.1, equine herpesvirus 2 protein E8, and the Kaposi's sarcoma-associated virus protein K13. The conserved module RXDLL is boxed.

MC159 and MC160 are closely related proteins from the molluscum contagiosum virus (13). The NH2 termini of the 241-amino acid protein MC159 and the 371-amino acid protein MC160 contain two motifs homologous to DEDs present at the NH2 terminus of FADD and repeated in tandem within the prodomain of FLICE. The DED motif is also present within EHV2-encoded protein E8 (171 amino acids), Kaposi's sarcoma-associated herpesvirus-encoded protein K13 (139 amino acids), and bovine herpesvirus 4-encoded protein E1.1 (182 amino acids). Each DED of these viral proteins contains a highly conserved module RXDL/I(L).

The presence of DEDs within E8, MC159, and MC160 suggests that these viral proteins might potentially antagonize the FADD-FLICE interaction and thereby attenuate TNFR-1- and CD-95-mediated apoptosis. Indeed, overexpression of MC159 significantly inhibited TNFR-1and CD-95-induced cell death (Fig. 10). The degree of inhibition was substantially greater than that achieved with the catalytically inactive dominant-negative version of FLICE and comparable in potency with CrmA. MC160 also inhibited TNFR-1- and CD-95-induced cell death (Fig. 10B), as did E8 (Fig. 10C).

Fig. 10



MC159, MC160, and E8 inhibit TNFR-1 and CD-95 induced apoptosis.
Overexpression of MC159 (A), MC160 (B), and E8 (C) inhibit CD-95 and TNFR-1 induced cell death. 293 or 293-EBNA cells were cotransfected with indicated plasmid together with the reporter construct pCMV β-galactosidase. Cells were fixed and stained 14-30 h following transfection. The data shwon are the percentage of blebbing blue cells as a function of total number of blue cells counted.

Additional studies were undertaken to delineate the point at which MC159 and E8 were exerting their inhibitory effect on the TNFR-1- and CD-95-induced death pathways. Both MC159 and E8 significantly blocked both TRADD and FADD killing, suggesting that these inhibitors must function downstream of these adapter molecules. In contrast, MC159 and E8 did not inhibit FLICE-induced death, suggesting that they must act upstream of active FLICE.

Binding studies were undertaken to investigate the potential mechanism utilized by E8 and MC159 to attenuate TNFR-1- and CD-95-induced cell death. In vitro and in vivo studies revealed that MC159 had a strong affinity for the DED of FADD while E8 preferentially bound FLICE.

We propose therefore that these viral DED containing proteins function by disrupting the FLICE activation complex.

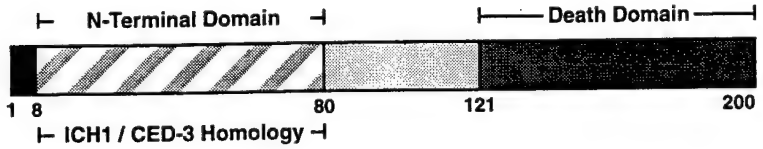
4) Molecular cloning and characterization of RAIDD a new "death' adapter molecule

The discovery of the adapter molecule FADD (2) was instrumental for understanding the very proximal events that occur after FAS receptor activation.

We were able to identify a second death adapter molecule RAIDD (for RIP-associated ICH-1/CED-3-homologous protein with a death domain). RAIDD was identified by searching the EST data bank for death domain motifs. The death domain (DD) is like the DED (death effector domain) a homophilic interaction motif. It is present on the cytoplasmic domain of several apoptosis inducing receptors like FAS TNFR-1 etc. Intriguingly, RAIDD not only contains a DD but an N-terminal domain found in some large prodomain Caspases like ICH-1 (Caspase-2 and CED3) (Fig. 10). This domain has recently been named CARD for Caspase recruitment domain (7).

Fig. 11

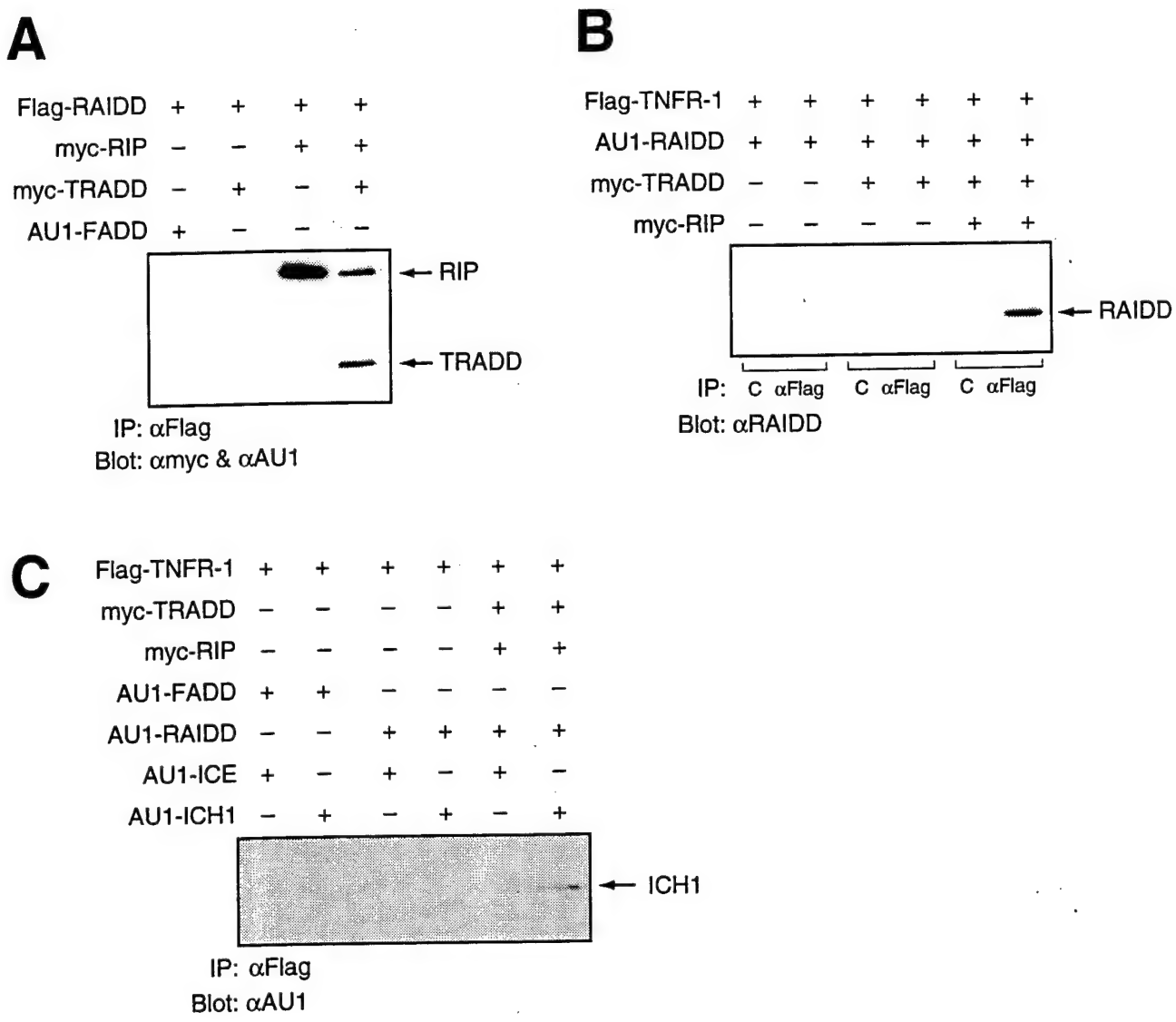
```
  1 MEARDKQVLRSLRLELGAEVLVEGLVLQYLYQEGILTENHIQEINA
 47 QTTGLRKTMLLLDILPSRGPKAFDTFLDSLQEFPWVREKLKKAREE
 93 AMTDLPAGDRLTGIPSHILNSSPSDRQINQLAQRLGPEWEPMVLSL
139 GLSQTDIYRCKANHPHNVQSQVVEAFIRWRQRFGKQATFQSLHNGL
185 RAVEVDPSLLLHMLE
```



Deduced amino-acid sequence of RAIDD and schematic represenation of the domain structure.

We were able to establish that the CARD domain is a third homophilic interaction domain that mediated death signal transduction. It bound to the homotypic prodomains of ICH-1 and CED3. Further, we established that the death domain of RAIDD selectively only interacted with the death domain of RIP and not FADD, TRADD (Fig. 11 a). We postulate that RAIDD is a component of a multiprotein complex that recruits ICH-1 to the TNF receptor. Experimental evidence was obtained in 293 expression system (Fig 12).

Fig. 12



On overexpression, RAIDD and ICH-1 are recruited to the TNFR-1 signalling complex.
a-c. 293 cells were transfected with the indicated combination of expression vectors. Detergent extracts of cells immunoprecipitated with either control mouse IgG or anti-Flag mAb. Co-precipitating proteins were analysed by immunoblotting with anti-epitope tag antibody (anti-Myc and anti-AU1 in a; anti-AU1 in c: or RAIDD antipeptide antibody in b).

TNFR-1 complexed with RAIDD only in the presence of TRADD and RIP (Fig. 12 b)., and through RAIDD, ICH-1 was recruited to the signaling complex (Fig. 12c).

Functional evidence that RAIDD is participating in the apoptosis branch of TNFR-1 signaling was demonstrated by its ability to induce apoptosis upon overexpression in MCF-7 cells. This apoptotic activity was inhibitable by z-VAD, CrmA and most significantly by the active site mutant of ICH-1.

What emerges from the functional analysis of the RAIDD adapter is the existence of an alternative pathway in TNFR-1 death signaling. At this point is unclear why this pathway and the TRADD-FADD-FLICE pathway are both engaged by the same receptor. It could be that each pathway is used specifically by different cells and tissues.

Conclusions:

The notion that Caspases are an integral part of the death effector arm was well established a year ago. Because Caspases are proteases they were generally not thought to be involved in the receptor proximal events. This bias, stemming from results obtained with receptor tyrosine kinases, was shattered by the discovery of FLICE. We have shown that FLICE is a genuine Caspase and that it directly associates with the FAS receptor. This discovery emphasizes the importance of the Caspase family of proteases not just as effector molecules but also as signaling molecules.

Our investigations of substrate specificity of FLICE support the idea of FLICE being at the apex of the FAS induced Caspase cascade. Therefore, FLICE becomes a prime target for the development of Caspase inhibitory drugs.

The activation mechanism of FLICE we elucidated using an experimental system by which FLICE can be activated in vivo by simply adding a dimerizing drug FK1012 H2. The zymogen form of FLICE has enough residual proteolytic activity to promote self processing at the high local concentrations obtained after FAS crosslinking.

We found a second member of the FLICE family of Caspases (FLICE2) that is also able to interact via the adapter molecule FADD with FAS and TNFR-1 receptors. Death signaling from these receptors is inhibited by dominant negative versions of FLICE2. Experiments are in progress to examine if FLICE activation involves FLICE2 and vice versa after co-recruitment to the FAS receptor. The possibility that FLICE2 is involved in signaling form other death receptors is also being investigated.

The importance of the FLICE activation step is exemplified by our discovery of viral DED containing proteins that target this step. The molluscum contagiosum virus relies fully on the inhibition of this step by MC159 and MC160 to evade host defense mechanism. This new class of viral inhibitors together with the known viral apoptosis inhibitors like CrmA, p35, E1B add up to a full palette of reagents that can be used as tools to identify what part of the apoptotic machine is used for a particular apoptotic stimulus (e.g. p53, growth factor withdrawal etc.).

The existence of additional pathways that involve recruitment of Caspases to receptors is indicated by the discovery of the adapter molecule RAIDD. TNFR-1 has two ways of recruiting Caspases. The FLICE family of Caspases is recruited to TNFR-1 by the TRADD and FADD adapters, while ICH-1 (Caspase-2) is recruited via RAIDD, RIP, and TRADD. At this point it is unclear if these are redundant pathways or if they are operating in different cells.

Publications supported by this Grant:

Duan H, Dixit VM: **RAIDD is a new 'death' adapter molecule.** *Nature* 1997, **385:**86-9.

Hu S, Vincenz C, Buller M, Dixit VM: **A novel family of viral death effector domain-containing molecules that inhibit both CD-95- and tumor necrosis factor receptor-1-induced apoptosis.** *J Biol Chem* 1997, **272:**9621-4.

Muzio M, Chinnaiyan AM, Kischkel KC, O'Rourke K, Shevchenko A, Ni J, et al.: **FLICE, A Novel FADD Homologous ICE/CED-3-like Protease, Is Recruited to the CD95 (Fas/APO-1) Death-Inducing Signaling Complex.** *Cell* 1996, **85:**817-827.

Muzio M, Salvesen GS, Dixit VM: **FLICE induced apoptosis in a cell-free system. Cleavage of Caspase.** *J Biol Chem* 1997, **272:**2952-6.

Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM: **An induced proximity model for FLICE (Caspase-8) activation.** *Science* 1997, **Submitted:**

Vincenz C, Dixit VM: **Fas-associated death domain protein interleukin-1beta-converting enzyme 2 (FLICE2), an ICE/Ced-3 homologue, is proximally involved in CD95- and p55-mediated death signaling.** *J Biol Chem* 1997, **272:**6578-83.

Meeting abstracts:

AACR conference on apoptosis: October 1996, Lake George, N.Y.

Engagement of CD95 or tumor necrosis factor 1 receptor (TNFR-1) by ligand or agonist antibodies is capable of activating the cell death program, the effector arm of which is composed of mammalian interleukin-1 converting enzyme (ICE)-like cysteine proteases (designated Caspases) that are related to the Caenorhabditis elegans death gene, CED-3. Caspases, unlike other mammalian cysteine proteases, cleave their substrates following aspartate residues. Furthermore, proteases belonging to this family exist as zymogens that in turn require cleavage at internal aspartate residues to generate the two-subunit active enzyme. As such, family members are capable of activating each other. Remarkably, both CD95 and TNFR-1 death receptors initiate apoptosis by recruiting a novel ICE/CED-3 family member, designated FLICE/MACH, to the receptor signaling complex. Therefore, FLICE/MACH represents the
apical triggering protease in the cascade. Consistent with this, recombinant FLICE was found capable of proteolytically activating downstream Caspases. Furthermore, CrmA, a pox virus-encoded serpin that inhibits Fas and tumor necrosis factor-induced cell death attenuates the ability of FLICE to activate downstream Caspases.

Keystone meeting on apoptosis: February 1996, Tamarron, CO.

The pivotal discovery that FLICE/MACH was recruited to the CD95 signaling complex by virtue of its ability to bind the adapter molecule FADD established that this protease has a role in initiating the death pathway (Boldin, M. P. et al. (1996) *Cell* 85, 803-815; Muzio, M., et al. (1996) *Cell* 85, 817-827). In this report, we describe the cloning and characterization of a new member of the Caspase family, a homologue of FLICE/MACH, and Mch4. Since the overall architecture and function of this molecule is similar to that of FLICE, it has been designated FLICE2. Importantly, the carboxy terminal half of the small catalytic subunit that includes amino acids predicted to be involved in substrate binding, is distinct. We show that the pro-domain of FLICE2 encodes a functional death effector domain that binds to the corresponding domain in the adapter molecule FADD. Consistent with this finding, FLICE2 is recruited to both the CD95 and p55 tumor necrosis factor receptor signaling complexes in a FADD-dependent manner. A functional role for FLICE2 is suggested by the finding that an active site mutant of FLICE2 inhibits CD95 and TNF receptor mediated apoptosis. FLICE2 is therefore involved in CD95 and p55 signal transduction.

References:

Belshaw PJ, Spencer DM, Crabtree GR, Schreiber SL: **Controlling programmed cell death with a cyclophilin-cyclosporin-**. *Chem Biol* 1996, **3:**731-8.

Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM: **FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis**. *Cell* 1995, **81:**505-12.

Chinnaiyan AM, Tepper CG, Seldin MF, O'Rourke K, Kischkel FC, Hellbardt S, et al.: **FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis**. *J. Biol. Chem.* 1996, **271:**4961-4965.

Crabtree GR, Schreiber SL: **Three-part inventions: intracellular signaling and induced proximity**. *Trends Biochem Sci* 1996, **21:**418-22.

Fearnhead HO, Dinsdale D, Cohen GM: **An interleukin-1 beta-converting enzyme-like protease is a common**. *FEBS Lett* 1995, **375:**283-8.

Freiberg RA, Spencer DM, Choate KA, Peng PD, Schreiber SL, Crabtree GR, et al.: **Specific triggering of the Fas signal transduction pathway in normal**. *J Biol Chem* 1996, **271:**31666-9.

Hofmann K, Bucher P, Tschopp J: **The CARD domain: a new apoptotic signalling motif**. *Trends Biochem Sci* 1997, **22:**155-6.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, et al.: **Cytoxicity-dependent APO-1(Fas/CD95)-Associated Proteins (CAP) form a Death-Inducing Signalling Complex (DISC) with the Receptor**. *EMBO* 1995, **14:**5579-5588.

Martin SJ, Newmeyer DD, Mathias S, Farschon DM, Wang H, Reed JC, et al.: **Cell-free reconstitution of Fas-, UV radiation-, ceramide-induced apoptosis**. *EMBO* 1995, **14:**5191-5200.

Pronk GJ, Ramer K, Amiri P, Williams LT: **Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER**. *Science* 1996, **271:**808-810.

Quan LT, Tewari M, O'Rourke K, Dixit VM, Snipas SJ, Poirier GG, et al.: **Proteolytic activation of the cell death protease Yama/CPP32 by granzyme B**. *PNAS* 1996, **93:**1972-1976.

Rotonda J, Nicholson DW, Fazil KM, Gallant M, Gareau Y, Labelle M, et al.: **The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis**. *Nature Structural Biology* 1996, **3:**619-625.

Senkevich TG, Bugert JJ, Sisler JR, Koonin EV, Darai G, Moss B: **Genome sequence of a human tumorigenic poxvirus: prediction of**. *Science* 1996, **273:**813-6.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, et al.: **Yama/CPP32ß, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase.** *Cell* 1995, **81:**801-809.

Walker NPC, Talanian RV, Brady KD, Dang LC, Bump NJ, Ferenz CR, et al.: **Crystal Structure of the Cysteine Protease Interleukin-1ß-Converting Enzyme: A $(p20/p10)_2$ Homodimer.** *Cell* 1994, **78:**343-352.

Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T, et al.: **Femtomole sequencing of proteins from polyacrylamide gels by nano-.** *Nature* 1996, **379:**466-9.